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**Small Interfering RNA and the Quest for an Effective Respiratory Syncytial Virus Therapy**Hudu, S.A.<sup>1\*</sup> and Hamal, N.S.<sup>2</sup>

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**Author for Correspondence\***: hudu.shuaibu@udusok.edu.ng/+234-803-909-9312**Summary**

*Respiratory syncytial virus (RSV) is the primary cause of lower respiratory tract infections among infants and children globally. Many vaccinology approaches have been assessed in rodent and primate models, however, no vaccines have been approved and there are limited effective antiviral agents available. This review aims to critically analyse the potentials of small interfering RNA as alternative treatment approaches in RSV. Online databases, both freely accessible and subscription based were searched through the University of Putra Malaysia's library. The search was limited to full research articles, review articles and meta-analyses, and articles were selected based on their relevance. A total 1352 articles were screened, however, only 823 were found to be relevant for this review and 65 have been cited here. Small interfering RNA is advantageous for developing antiviral treatments because of its ability to acts at the nucleotide level. This provides information needed to design therapeutic RNAi, which can subsequently accelerate the rate at which potential therapies enter the drug development industries. The antiviral effect of RNAi usually occurs through specific biochemical inhibition of viral protein function. The main components in RNAi drugs have proved efficient at inhibiting RSV, and multiple siRNA can be designed to silence the targeted viral genes and target the sites of infection.*

**Keywords:** Antiviral; Gene silencing; Respiratory syncytial virus; small interfering RNA; Therapy

**Introduction**

Respiratory syncytial virus (RSV) is an enveloped, negative-strand and non-segmented RNA virus. The World Health Organization (WHO) and the Centre for Disease Control and Prevention have reported that this virus is the primary cause of lower

respiratory tract infections (LRTI) in infants and children globally. Approximately 80% of infants are infected with RSV during their first year of life with a peak incidence between 2 and 6 months. Fever, coughing, coryza, expiratory wheezing and respiratory distress are characteristic features of RSV that causes upper and LRTIs. Severe RSV infections could develop into bronchiolitis and pneumonia which may eventually lead to death. Reinfection normally occurs due to low levels of serum neutralizing antibodies produced by the RSV protection mechanism. Direct contact with secretions and fluids from infected bodies is the mode of transmission for this infectious virus. Thus frequent hand washing, isolation of infected patients and contact precautions remain the best preventative actions.

Aerosolized ribavirin, a specific antiviral agent, has been used in patients with severe RSV infections; however, its ability to reduce hospitalization and mortality has not been proven and therefore its use is limited. Apart from the high costs of administering this drug, the long-term side effects on health care workers remain unknown, especially its effect during pregnancy. A novel compound called RSV604 is currently in phase II clinical trials. It is a benzodiazepine with sub-micromolar anti-RSV activity and is active against clinical isolates. Palivizumab, a monoclonal antibody that targets the viral F protein by recombinant DNA technology, has been used as a prophylaxis agent against RSV infections. It is recommended for use in high risk infants with chronic lung disease or congenital heart disease, especially those born prematurely. Intramuscular administration of palivizumab once per month during the RSV season has been shown to reduce the risk of hospitalization by 45%–55%.

As the vaccine approach remains a long way off, and with the limited availability of efficient

antiviral agents, identifying alternative RSV treatments is of great importance. The use of RNA interference (RNAi) is a good strategy for fighting viral infections as RNAi are natural defence mechanisms that the innate immune system uses to fight viruses. Therefore, this review aims to critically analyse the potentials of small interfering RNA as alternative treatment approaches in RSV infection.

### **Methodology**

The search for refereed journal articles was performed using the University of Putra Malaysia's online subscription databases using Medline, SCOPUS and Google Scholar. All publications were in English; duplicates, abstracts, conference proceedings, comments and short communications were excluded. The original search result gave 1352 results of which only 823 were found to be relevant for this review. Of these, 67 articles were selected based on their relevance to the topic under review

### **Respiratory Syncytial Virus**

RSV is a pleomorphic, enveloped, non-segmented and cytoplasmic virus that contains single-stranded, negative-sense RNA. The RNA is associated with viral proteins, consisting of a nucleocapsid core wrapped within a lipid envelope. RSV belongs to the Paramyxoviridae family and is classified in the Pneumovirus genus. There are two Paramyxoviridae subfamilies: Paramyxovirinae, which includes measles, mumps and parainfluenza; and Pneumovirinae, which includes most of the RSV viruses (e.g. human RSV, bovine RSV and metapneumovirus). Distinctive characteristics of Pneumovirus that differentiate it from other members of the Paramyxoviridae family are the presence of the second matrix protein (M2) and enveloped proteins without hemagglutinin or neuraminidase activity.

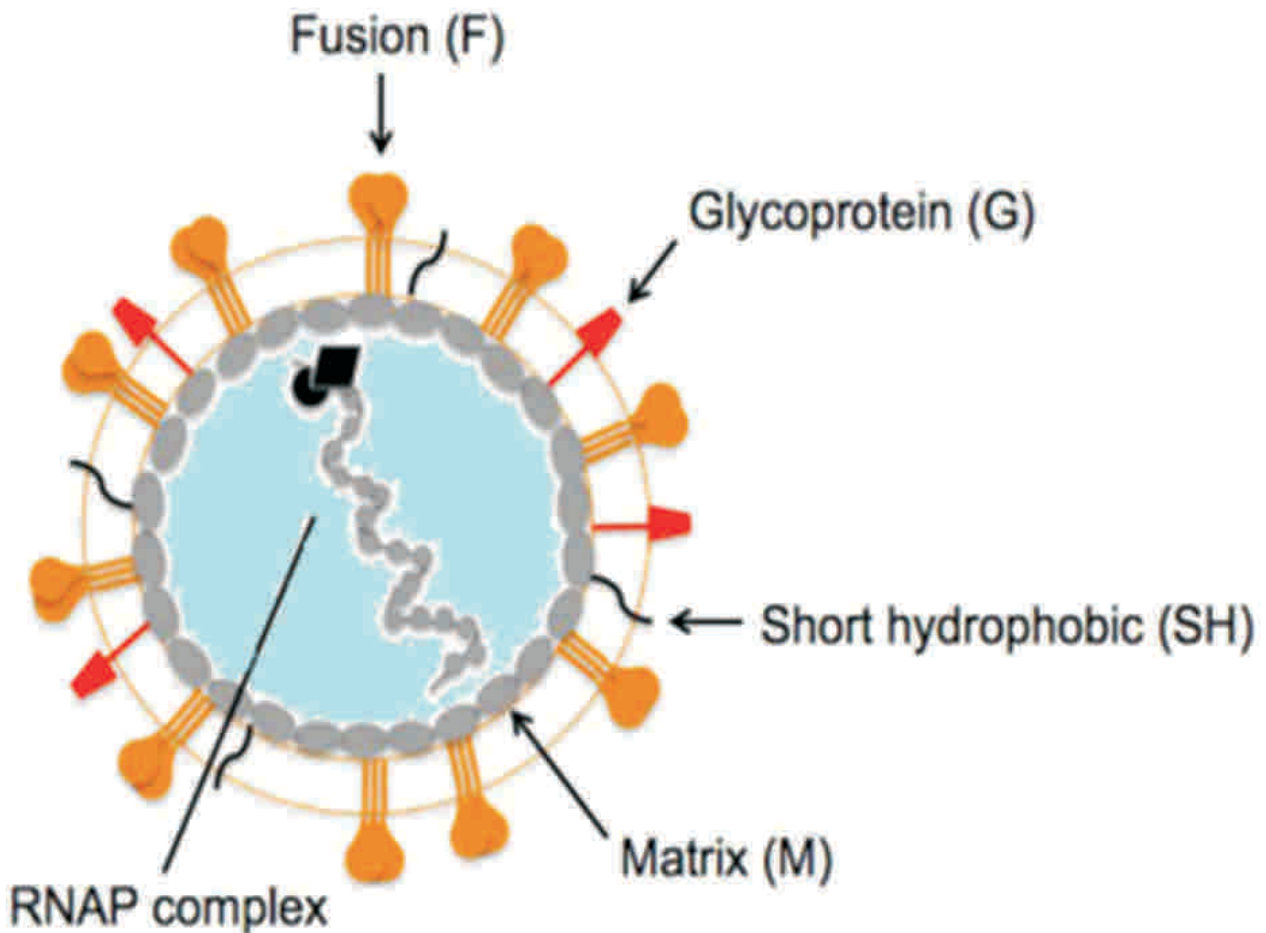
RSV was first isolated from a group of flu-affected chimpanzees in 1956. 'Chimpanzee coryza agent'

was named following the recovery of a cytopathic agent from these chimpanzees. Remarkably, humans who made direct contact with the chimpanzees were also infected with the virus and showed symptoms of coryza and acute upper respiratory tract illness, although this was reported to be less chronic than in the chimpanzees. The same virus was subsequently isolated from children with respiratory illness. Serologic studies indicated that the infection in infants and children was common; however, in a paediatric setting, RSV was found to be the main pathogen causing serious respiratory illness globally.

There are two subtypes of RSV: antigenic subtype A (RSV-A) and antigenic subtype B (RSV-B). These subtypes are differentiated via their molecular genetic differences and the reaction of monoclonal antibodies against the G and F glycoproteins. It has also been shown that strains of subtype A are more virulent and usually more predominant than subtype B strains, and both virus subtypes cocirculate. However, the predominant strains vary across different locations and may shift from year to year. Variation in the circulation patterns of RSV strains helps them to evade immunity; for example, the strain cluster of subtype A and B. The variability of this viral strain also helps it to alter the pathogenicity and action of the virus which later leads to repeated infections and outbreaks

### **Virus Protein**

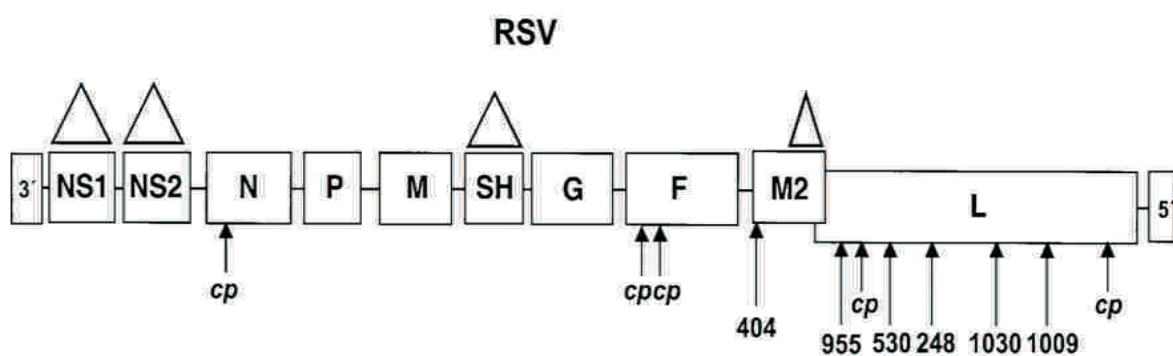
The phenotypic profile of RSV is characterized by the number of genes, the surface glycoproteins and the helix diameter. The genome encodes 11 proteins: nine structural and two non-structural. The large glycoprotein G (attachment protein) is a disulphide-bonded glycoprotein, and the F glycoprotein (fusion protein) is the major antigenic determinant of the virus (Figure 1). (Modified from Cox and Williams, 2013)



**Figure 1: Schematic morphology of RSV with various forms of viral protein.**

The G protein is responsible for virus attachment, whilst viral penetration and syncytium formation are mediated by the F protein. These two proteins are the only ones that induce neutralizing antibodies that cause an accumulation of mutations in response to host immunological pressure (Connors et al., 1991). The SH (small hydrophobic protein), M (matrix

protein) and M2 proteins are envelope-associated proteins; the N (nucleoprotein), P (phosphoprotein) and L (nucleoprotein) proteins are present in the RSV nucleocapsid; and NS1 and NS2 are non-structural proteins found in infected cells but not in the virion as shown in Figure 2. (Ruuskanen and Ogra, 1993).



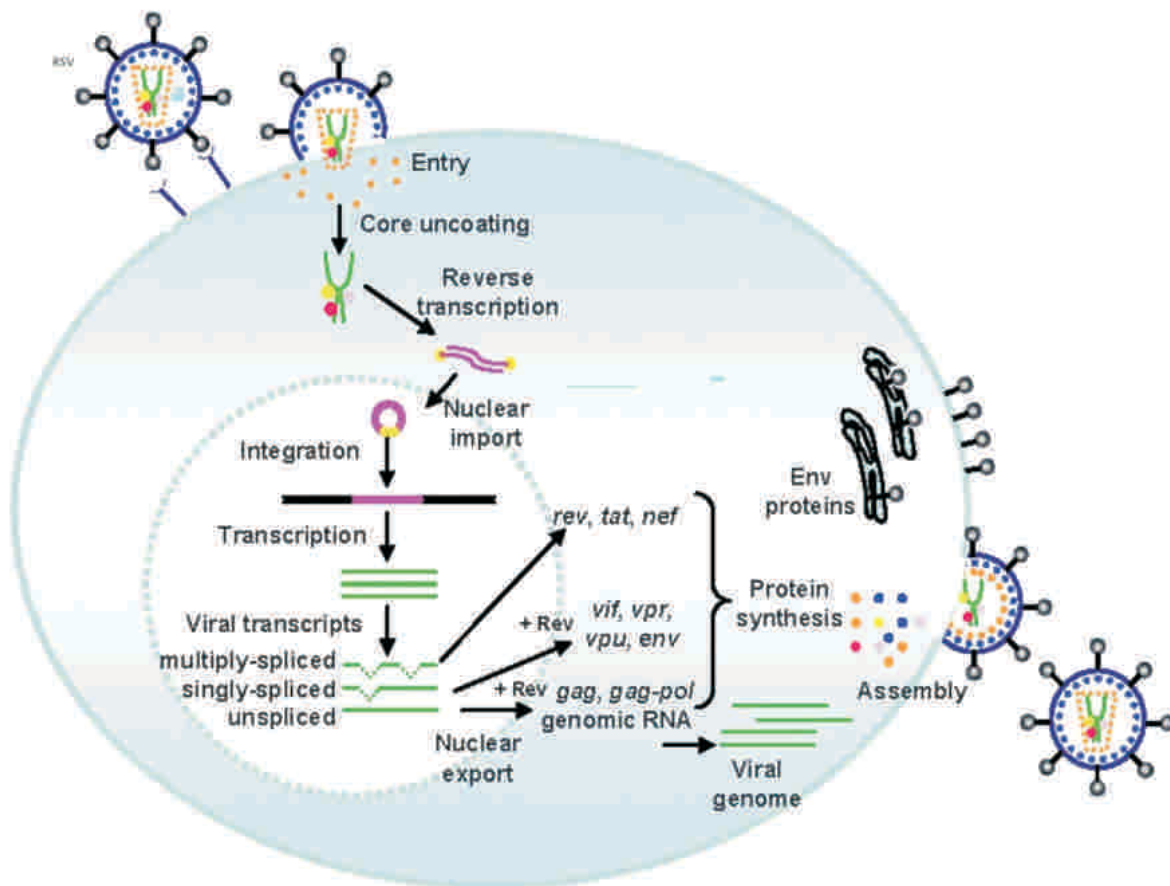
**Figure 2: Representation of Respiratory Syncytial Virus genome organization. The RNA genome encodes for 11 proteins; 9 structural and 2 non-structural**

The RSV M2-2 protein is a small (90 amino acids for strain A2) protein encoded by a second downstream open reading frame (ORF) in the M2 mRNA which slightly overlaps the 5' proximal M2-1 ORF. The M2-2 protein is responsible for the regulatory 'switch' from transcription to RNA replication. It initially provides high levels of mRNA synthesis followed by the synthesis of RNA in favour of genomic RNA for virion assembly. Bermingham and Collins (1999)

found that recombinant RSV lacking M2-2 grows less efficiently than wildtype.

### Replicative Cycle

Viral entry occurs when the viral envelope fuses with the cell membrane. The initiation interaction occurs when the RSV G protein binds to a polysaccharide in the host cellular matrix that contains repeating disaccharide subunits (Figure 3).



**Figure 3: Viral life cycle. Pattern recognition receptors are able to target multiple steps in the viral life cycle (Modified from: Sakuma *et al.*, 2012).**

In addition to GAGs, a number of other cellular proteins have also been implicated in RSV infection *in vitro*, including intercellular adhesion molecule-1, RhoA, the CX3CR chemokine receptor and annexin II. Following the initiation interaction, the F protein interacts with RhoA to mediate virus attachment. Viral replication and gene expression occur in the cytoplasm where the nucleocapsid and genetic material are released. The M2-2 gene is responsible for the switch from transcription to the production of genomic RNA. The polymerase then enters the genome at its 3' end and the genes are transcribed into mRNA. A polar transcription gradient leads to the

more frequent transcription of genes at the beginning of the promoter compared with downstream genes. Replication serves as a template for genome synthesis as it generates an antigenome (e.g. a complete positive-sense RNA complements of the genome). The genome and antigenome must be coated with the N-protein in order for RNA synthesis to occur. Next, the M protein regulates the assembly of RSV by interacting with F, G and nucleocapsid proteins (N, P and M2-1). An envelope derived from the membrane is required by the newly synthesized protein, which leads to self-assembly and budding.



The viral mRNAs and proteins can be detected intracellularly within 4–6 hours of infection. The accumulation of mRNA plateaus at 14–18 hours. This apparent transcription shut-off may be due to the M2-2 protein, which appears to shift the balance of RNA synthesis from transcription to RNA replication. Using this hypothesis, Ruuskanen and Ogra (1993) concluded that the accumulation of M2-2 during infection increasingly favours RNA replication over transcription. The release of progeny viruses begins at 10–12 hours post infection reaches a peak after 24 hours and continues until the cells deteriorate at 30–48 hours.

### **Disease Associated with RSV**

RSV is recognized as the primary cause of respiratory tract infections in children less than 2 years old and the majority (80%–90%) of infants experience at least one RSV infection within the first 2 years of life. Primary RSV infection occurs early in life and is most likely to affect the lower respiratory tract. Infected body secretions and fluids are the mode of transmission for this infectious virus. Past RSV infections provide immunity against reinfection from viruses within the same subgroup; however, protection against natural infection is incomplete and is correlated with low levels of serum neutralizing antibodies. Symptoms of mild RSV respiratory infections are similar to those produced by the common cold, whilst severe cases of RSV infection cause symptoms such as expiratory wheezing, otitis media, bronchiolitis and pneumonia.

RSV is the primary cause of bronchiolitis in young children, whilst pneumonia is the most common feature in infected elderly patients. Severe symptoms of RSV-mediated LRTIs may be persistent and chronic compared to other viruses. Of all children hospitalised for bronchiolitis, pneumonia and tracheobronchitis, RSV is responsible for 50%–90%, 5%–40% and 10%–30% of admissions, respectively. Moreover, children with lung and heart disease are at high risk of contracting severe RSV infections that could lead to death.

### **Diagnosis**

Early diagnosis is advantageous because it allows isolation measures to be implemented to limit the spread of the infection (i.e. in a hospital setting or nursing home). Identification of the virus using cell culture practices remains the most reliable method. Specimens are best obtained by aspiration or gentle washing out of nasal or nasopharyngeal secretions or by nasal swabs. The nature of the virus requires that samples be kept cold, not freeze-thawed and be inoculated onto cell monolayer cultures sensitive to

RSV as quickly as possible. The development of RSV cytopathology requires 3–7 days of incubation; however, culture-based detection can be made to be more rapid and efficient through the use of immunofluorescence and shell vial culture. Rapid diagnostic methods involve the detection of viral antigens or nucleic acids in secretion samples.

In RSV enzyme-linked immunosorbent assay (ELISA), samples containing respiratory secretions are incubated briefly with an RSV-specific antibody. In direct immunofluorescent assays (DFA), secretions containing exfoliated cells are reacted with fluorescent RSV-specific antibodies and examined by microscopy; however, DFA requires specialized equipment (e.g. a fluorescence microscope) and greater expertise. RT-PCR assays are commercially available for RSV and the sensitivity of these tests can equal or exceed that of cell culture or antigen-based assays. For example, the sensitivity and specificity of RSV RT-PCR assays has been reported to be over 90%. Studies have shown that testing using RT-PCR is particularly advantageous for adults as the nasopharyngeal titre of infectious virus may be low despite illness. The N gene of RSV is often used as the target for nucleic acid-based assays because of its high level of conservation and transcription. Sequence polymorphisms that distinguish between RSV genotypes A and B have been used to design genotype-specific primers or probes for RSV genotyping.

### **Treatment and Prevention of RSV Infection**

There is currently no effective RSV antiviral treatment, therefore supportive therapy is still the basis of care for hospitalized children (i.e. administration of supplemental oxygen, mechanical ventilation and fluid replacement). However, ribavirin, the Food and Drug Administration (FDA) approved drug, has been used to manage complicated cases of RSV infection alongside intravenous immunoglobulin. The search for a preventative treatment is still ongoing with palivizumab being the only drug approved for use in selected patient populations in the United States. Additional vaccines that will help to reduce disease severity in infants and children are also being searched for. Ribavirin therapy has proved to be most beneficial in critically ill patients that rely on mechanical ventilation as well as in recipients of lung transplants; however, the aerosolization risk of ribavirin remains an issue. Alternative therapies using cathelicidins seem to be promising but require further evaluation in terms of their safety and dosing regimens. Studies involving probiotics and vitamin D are currently of interest and, if positive, will provide a relatively cheap and stress-

free option for children infected with RSV.

### **Vaccines Development**

In their list of vaccines that require development, the WHO had listed RSV as a priority due to its global high morbidity and mortality rates ; however, the RSV vaccine development progress has been delayed for several reasons. Obstacles faced when developing the RSV vaccine include the nature of the disease, the vaccine design and its infectivity in young patients. Vaccination is recommended in early life, ideally in infants 1 month old or younger, as the most infective patients are children below 6 months of age. However, new-borns and preterm infants may not respond favourably to immunization as their immune system is poorly developed . Frequent reinfection throughout life is another unfavourable observation of RSV infection . The low number of relevant animal models is one factor that has contributed to the failure of the vaccine development strategy.

Live attenuated RSV vaccines that can be administered intranasally would provide immunity in both upper and lower respiratory systems . However, the disadvantage of this type of vaccine is that vaccinated children with sufficient immunity may cause viral shedding, therefore unvaccinated children would be at risk of infection . Development of a live genetically engineered RSV vaccine remains a possibility .

Vaccine development research is ongoing with studies of the F and G glycoproteins. Subunit vaccines containing purified F and G glycoproteins or novel chimeras of F and G glycoproteins are being produced and may be appropriate for use in some high risk populations . Research is also being conducted into the feasibility of introducing plasmid cDNA encoding reporter-tagged virus genes into mammalian muscle tissue. These compounds have been found to be present for months after their initial introduction into the tissue . This prolonged effect may be useful for allowing an immune response to develop in new-borns as maternal antibody levels decline.

### **Choice of Genes and Disease to Target**

RNAi has had a great impact in treating diseases in which affected cells have RNAi machinery, as well as in diseases caused by the exaggerated expression of a dominant gene . Most studies have focused on diseases that have not been cured by current therapies, such as cancer, neurodegenerative diseases, viral infections and macular degeneration . Due to the antiviral response of RNAi, viral infection becomes a complex process especially in plants and lower organisms. Therefore, the potential for genetic

mutations to escape from RNAi needs to be taken into account during viral infection and cancer formation. A number of alternative studies have been carried out such as targeting multiple genes at once, targeting essential genes or targeting highly conserved sequences in which mutations would enhance viral fitness or tumour cell survival .

### **Advantages of RNAi**

Public health is currently seriously threatened by known and emerging viruses, and not all vaccines can provide protection against entire virus species. RNAi has been an outstanding candidate for clinical use as it has proven effective in in vitro studies and in animal models at low doses . RNAi has two major advantages for the development of antiviral treatments: Firstly, it acts at nucleotide level, which provides information needed to design therapeutic RNAi that can later accelerate the rate at which potential therapies enter drug development industries. Secondly, antiviral molecules usually act by specific biochemical inhibition of viral protein function by simultaneously interacts with a host factor that tends to cause detrimental side effects that could impact on patient health and compliance. To date, only mRNA has been identified as the target for RNAi during the replication of negative-strand RNA viruses such as influenza A, hepatitis delta virus, RSV influenza , hepatitis C coronavirus , herpes simplex virus 2 , hepatitis A , HIV and Ebola . These studies provide proof of principle that a novel class of RNAi-based prophylactic and therapeutic strategies can be used against acute and chronic viruses.

### **Conclusion**

In recent years, alternative approaches to inhibit human viruses using RNAi technology have continued to develop in both industry and academia. Small interfering RNAi (siRNA), the main components in RNAi drugs, have proved efficient at inhibiting RSV, and multiple siRNA can be designed to silence the targeted viral genes and target the sites of infection. RNAi drugs are effective regardless of the immune status, therefore this drug therapy is applicable for use in patients who are often susceptible to severe disease (i.e. young, elderly or immunocompromised individuals) caused by RSV infection.

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